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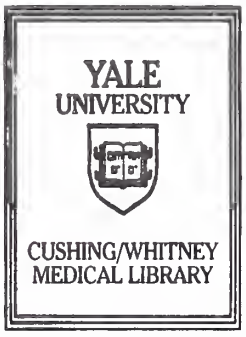
UNCOUPLING PROTEIN 2 EXPRESSION ARE NOT ASSOCIATED WITH ATHEROSCLEROTIC DISEASE SEVERITY

John Kiene Forrest

YALE UNIVERSITY

2005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UNCOUPLING PROTEIN 2 EXPRESSION AND ITS ASSOCIATION WITH ATHEROSCLEROTIC DISEASE SEVERITY

A Thesis Submitted to the

Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

John Kiene Forrest

2005

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UNCOUPLING PROTEIN 2 EXPRESSION AND ITS ASSOCIATION WITH ATHEROSCLEROTIC DISEASE SEVERITY. John K. Forrest, J. Dawn Abbott, Kerry S. Russell, and Raymond R. Russell. Section of Cardiology, Department of Internal Medicine, Yale University, School of Medicine, New Haven, CT.

Uncoupling proteins (UCPs) are inner mitochondrial membrane channels that dissipate the proton gradient generated by the respiratory chain, and have been shown to play an important role in attenuating reactive oxygen species (ROS) generation. Animal studies suggest that changes in UCP expression in circulating monocytes may effect atherogenesis by regulating the production of ROS known to cause endothelial damage. However, the association between mononuclear cell UCP expression in humans and coronary artery disease has not been assessed. The purpose of this study was to investigate the association between mononuclear cell uncoupling protein 2 (UCP2) expression and the severity of coronary artery disease in patients undergoing coronary angiography. Peripheral blood samples were obtained from 103 enrolled patients undergoing coronary angiography. Coronary atherosclerotic disease burden was assessed using a 15 segment stenosis scoring system. Messenger RNA was isolated from the peripheral mononuclear cells and real-time PCR was performed to evaluate UCP2 expression. In patients with severe coronary artery disease (stenosis score >25) there was a 35% decrease ($p<0.02$) in monocyte UCP2 mRNA expression compared to patients with no to mild coronary artery disease (stenosis score 0-10). There was no significant difference in the monocyte expression of UCP2 mRNA in patients with moderate disease (stenosis score 11-25) compared to patients with no to mild coronary artery disease. This study demonstrates that decreased monocyte UCP2 expression correlates with increased atherosclerotic disease burden in patients, and supports the hypothesis that changes in monocyte UCP2 expression may modulate vascular oxidant stress and play a role in atherogenesis.

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INTRODUCTION

Atherosclerosis is the leading cause of morbidity and mortality in Western society accounting for more deaths than all types of cancers combined (1). In 2000, there were 946,000 deaths in the United States that were attributed to cardiovascular disease, the primary manifestation of atherosclerosis (2). Although initially thought of simply as a lipid storage disease, atherosclerosis is now recognized to be a form of chronic inflammation, characterized by the progressive deposition of lipids and fibrous elements in large- and medium-sized arteries (1, 3-5). This inflammation, resulting from the interactions between lipoproteins, monocyte-derived macrophages, T cells, and native cellular elements of the arterial wall, leads to the proliferation of a variety of cell types which progressively narrow the lumen of blood vessels, impeding blood flow. The manifestations that result include angina from stenosis of coronary arteries, stroke from stenosis of the arteries of the cerebrovascular system, and claudication from stenosis of arteries in the peripheral circulation.

EPIDEMIOLOGY AND RISK FACTORS

Epidemiological studies over the past 25 years have demonstrated that the development of atherosclerotic disease is multifactorial with both genetic and environmental components. While some risk factors such as cigarette smoking are preventable and can be modified, many of the other proposed contributors to the development of atherosclerotic disease are not completely understood.

Age

In the clinical setting, one of the most important factors for determining the relative risk of atherosclerotic disease is age. For men living in the United States, the relative risk of developing cardiovascular disease is seven times greater for a 60-64-year-old than it is for a 30-

34-year-old; 21% versus 3% (6). The significant impact age has in risk assessment is evident in the components of the Framingham Heart Study's point system, formulated to predict the 10-year risk for developing cardiovascular disease. In this 14-point scoring system, up to 7 points can be attributed to age alone.

Gender

Another risk factor that is not subject to modification, but has been shown to contribute significantly to increased risk of cardiovascular disease is gender. Investigation into the reason for this initially focused on estrogens and their "protective" effects, based on the fact that the incidence of cardiovascular disease increases substantially in women after menopause. However, recent studies have demonstrated that estrogen supplementation may actually increase the risk of cardiovascular disease in postmenopausal women (7), although these studies may suffer from methodologic problems. Other explanations for the difference in the incidence of atherosclerotic disease between men and women suggest that women are protected due to the fact that women have, on average, a higher concentration of high-density lipoproteins than men (1). However, even when such factors are accounted for, multiple studies have shown that men have a greater risk of developing heart disease than do age-matched premenopausal women (8).

Cigarette Smoking

While having been promoted early in the last century as a practice supported by physicians, as depicted in the Fatima Cigarette advertisement published in the August 11, 1930 edition of Time Magazine whose caption reads: 'The old doctor handed over to his doctor son a family tradition of high ideals and devoted service. And another tradition has survived, too . . . Fatima Cigarettes', and even suggested as being helpful for those students writing their thesis (see figure 1), cigarette smoking has long been known to increase the incidence of coronary artery disease (9-11).

More recent data, including the Surgeon General's report, estimates that smoking may increase atherosclerotic disease and the risk of heart attack by as much as 50% (12). Of interest is the fact that the risk of myocardial infarction in ex-smokers approaches that of non-smokers just 2 years after smoking cessation (13).

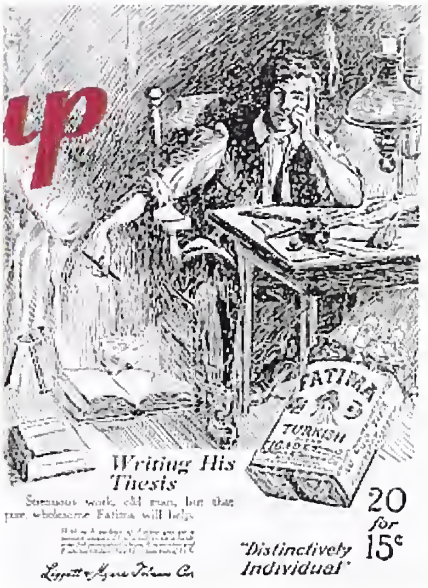

Figure 1. Early 20th Century Ad.
Smoking advertisement from the Pennsylvania Gazette, April 5th, 1913, showing a graduate student writing his thesis. The caption underneath reads: 'Strenuous work, old man, but that pure, wholesome Fatima will help'.

Hypertension

Defined as a systolic pressure >140 mm Hg or a diastolic pressure >90 mm Hg, hypertension affects more than 75% of people over the age of 75 (14). These individuals are at increased risk for the development of cardiovascular disease and studies have shown that there is a linear relationship between increased blood pressure and the incidence of atherosclerotic disease (15). As with smoking, multiple studies have revealed that the treatment of hypertension dramatically reduces the risk of coronary heart disease and myocardial infarction. These studies have shown that successful treatment of hypertension results in a 24-31% reduction in the five-year risk of developing symptomatic coronary heart disease (16-19).

Serum Cholesterol

The association between low-density lipoprotein (LDL) concentration and atherosclerosis is most evident in patients with the autosomal dominant disorder familial hypercholesterolemia. This disorder, which is caused by a defective LDL receptor, leads to a 2- to 5-fold elevation in LDL concentrations in heterozygous individuals, and a 4- to 6-fold increase in LDL concentrations in homozygous individuals. By the age of 15, a remarkable 85% of homozygotic patients will have suffered a heart attack; in heterozygotic patients the age at which 85% of individuals will have had a myocardial infarction is 60 years (20). However, the LDL concentration need not be elevated so dramatically to have a significant effect on atherosclerotic disease, as there is a linear relationship between LDL concentration and atherosclerosis for all levels of LDL.

As opposed to LDL, for which there is a positive association between serum concentrations and atherosclerosis, the relationship between high-density lipoprotein (HDL) concentrations and atherosclerosis is inverse (21). Thus while increased LDL concentrations, as seen in familial hypercholesterolemia, lead to early atherosclerosis, the absence of HDL cholesterol can also lead to early atherosclerosis, as is seen in some families with Tangier disease, an autosomal recessive disorder characterized by the absence of HDL cholesterol due to a defect in the ATP binding cassette transport-1 protein (22-26). Newer pharmacological therapies have focused on the role of HDL cholesterol by attempting to not only lower LDL, but also raise HDL concentrations.

Diabetes

Now considered to be an epidemic in the United States, diabetes affects 6.2% of the American population (approximately 17 million people) (27). For these patients, the risk of developing coronary artery disease is three to five times greater than the general population (28). While other confounding conditions, including hypertension and hyperlipidemia are more

prevalent in patients suffering from diabetes than in the general population, it has been shown that no more than 25% of the excess coronary atherosclerosis risk from diabetes can the attributed to these other risk factors (28-30).

Family History

The critical importance of obtaining a complete history from each patient is demonstrated by studies that have shown that coronary artery disease, when it has occurred before the age of 60 in first degree relatives, is a strong independent risk factor for cardiovascular events (31, 32). While often associated as a risk factor in patients who already are at increased risk for the development of coronary artery disease, it is important to recognize that even within low- and intermediate risk groups, individuals with a family history of premature coronary artery disease have a significantly higher relative risk than individuals without a positive family history (31). When compared with men with no parental history of myocardial infarction (MI), patients with a history of maternal MI only, paternal MI only, and both maternal and paternal history of MI have a relative risk of 1.71, 1.40, and 1.85 respectively. Among women, the respective relative risks are 1.46, 1.15, and 2.05 (33).

C-Reactive Protein

Recent studies have demonstrated that there is a linear relationship between serum concentrations of the inflammatory marker C-reactive protein (CRP) and the risk of myocardial infarction or death from coronary causes. Additionally, CRP concentrations have been shown to correlate inversely with the progression of angiographically documented coronary atherosclerotic disease when patients are treated with statins, independent of change in LDL concentrations (34, 35). Other markers of inflammation, including homocysteine, have also been implicated as independent risk factors for the development of coronary heart disease (36), and the investigation

into the molecular mechanisms behind these relationships has offered new insights into the pathogenesis of atherosclerosis and the important role that inflammation plays in this process.

THEORIES OF PATHOGENESIS OF ATHEROSCLEROSIS

In humans, the first lesions of atherosclerosis begin with the formation of the 'fatty streak', which can usually be seen in the aorta as early as the first decade of life, in the coronary arteries in the second decade, and in the cerebral arteries by the third or fourth decade (4). While not clinically relevant, fatty streaks represent the foundation upon which atherosclerotic lesions will continue to develop. The fatty streak occurs as the result of an increase in the concentration of lipoproteins within the intimal layer of the arterial vessel wall. There are several proposed mechanisms to explain this accumulation of lipoproteins, and while they are similar in many aspects, they each emphasize different concepts as the necessary and sufficient events to support the development of atherosclerotic lesions.

Response to Injury Model

The first proposed theories on the 'atheromatous effection of arteries,' date back to the 1850s, and include Rokitansky's "incrustation" hypothesis, and Virchow's "lipid transudation" hypothesis. While remarkable in that they were correct in identifying that fibrin deposition and modified lipid complexes caused atherosclerosis, these first theories saw the disease as involving passive deposition rather than active inflammation (37, 38). The recognition that there was an inflammatory component to atherosclerosis was not suggested until over 100 years later when Ducan discovered that "injury to the endothelium causes insudation of inflammatory fluid from the plasma into the intima followed by degeneration and proliferation" (39). This response-to-injury hypothesis was modified and expanded upon by Ross and Glomset who suggested that "local injury to the endothelium increases the concentration of plasma proteins in the vicinity of

medial smooth muscle cells, and in response to some of these proteins, the cells migrate into the intima and proliferate" (40-42).

This initial response-to-injury theory, however, suggested that direct and continued disruption or desquamation of the endothelium was the principal step necessary for atheromatous formation and progression. More recent studies have demonstrated that the endothelium covering atherosclerotic plaques is often completely intact, and thus the hypothesis has been modified to suggest that endothelial dysfunction alone is sufficient to initiate atherogenesis through increased permeability to atherogenic lipoproteins (1, 43). However, other studies have shown that increased endothelial permeability to atherogenic lipoprotein alone does not fully explain atherosclerotic plaque formation. The reason for this is that in the normal artery, the rate of LDL entry is often greater than the rate of LDL accumulation, suggesting that endothelial dysfunction is not required for LDL entry into the arterial wall (44). Furthermore, histologic examination of coronary arteries has shown that atherogenesis does not occur uniformly throughout the vessel, but rather at specific predisposed regions (45, 46). Because LDL entry through the endothelium is relatively homogeneous throughout the vessel, it is possible that atherogenic lipoprotein entry into the arterial wall may not depend on endothelial injury or dysfunction. Given this, two additional models have been proposed that are currently under active investigation. These are referred to as the response-to-retention model and the oxidative modification model (1).

Response to Retention Model

This hypothesis, first proposed by Williams and Tabas (47), suggestes that lipoprotein retention within the arterial wall is the event necessary for atherosclerotic plaque formation. This retention is proposed to be facilitated by Apolipoprotein B-100, the only protein associated with LDL, which is retained within the arterial wall in close association with arterial proteoglycans (1, 48, 49). Studies investigating the retention of apoplipoprotein B-containing liproproteins have demonstrated that the binding of these molecules to proteogycans is important in the early stages

of atherogenesis (50). Once retained within the arterial wall, LDL can form micoaggregates, which are actively taken up by macrophages and smooth muscle cells, resulting in foam cell formation and thus initiating atherosclerotic plaque formation (51, 52).

The Oxidative Modification Model

The oxidative modification hypothesis focuses on the singular importance of LDL in atherogenesis, but is unique in that it suggests that LDL in its native state in not atherogenic, and that chemical modifications are necessary for LDL particles to be taken up by macrophages in the initiating phase of atherosclerosis. The recognition that uptake of LDL by macrophages requires that the LDL particle be chemically altered before it can be internalized by macrophages via a "scavenger receptor" pathway was first made by Goldstein et al. in 1979 (53). Since then, it has been determined that these LDL modifications include oxidation and nonenzymatic glycation. Nonenzymatic glycation of apolipoproteins and other arterial vessel wall proteins has been shown to occur in diabetic patients with sustained hyperglycemia, one of the factors which may contribute to the increased incidence of coronary disease in this population. The role of oxidative modification is more clearly understood than the role of nonenzymatic glycation, as oxidation of LDL converts the previously non-atherogenic LDL particle into one capable of uptake by macrophages (figure 2). Upon LDL oxidation, apolipoprotein B-100 (apo B) undergoes a chemical modification whereby it increases the net negative charge of the LDL particle (54). This increase in negative charge enables macrophages to internalize the LDL particle via a number of scavenger receptor pathways (55). These LDL-containing macrophages become cholesterol ester-rich foam cells, forming the 'fatty streak' and the beginning of atherosclerotic plaque formation.

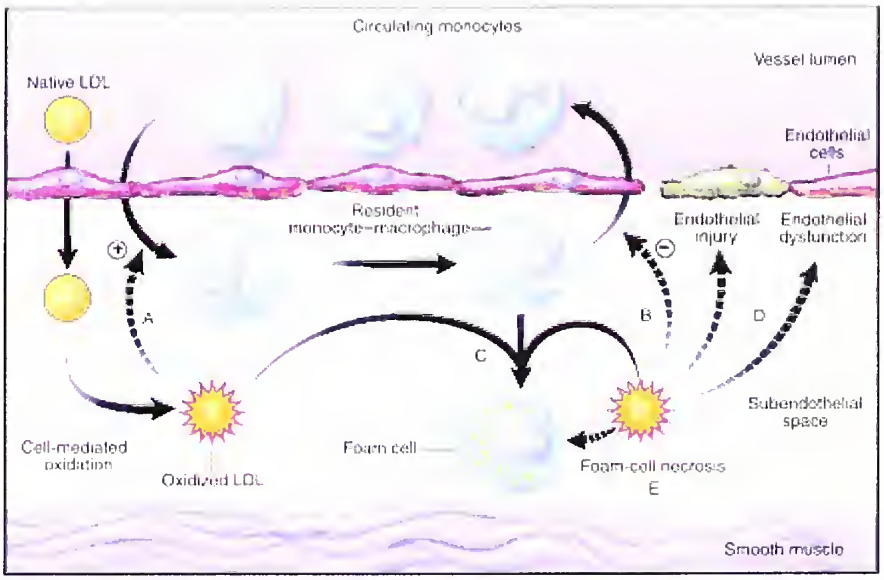

Figure 2. Oxidative modification hypothesis of atherosclerosis.
LDL becomes entrapped in the subendothelial space where it is subject to oxidative modification by resident vascular cells such as smooth muscle cells, endothelial cells, and macrophages. Oxidized LDL stimulates monocyte chemotaxis (*A*), prevents monocyte egress (*B*), and supports foam cell formation (*C*). Once formed, oxidized LDL also results in endothelial dysfunction and injury (*D*), and foam cells become necrotic due to the accumulation of oxidized LDL (*E*). *Figure from Diaz et. al (56).*

Other atherogenic processes are also initiated in the aftermath of LDL oxidation, and not all of these require changes to apo B. For example, it has been shown that during the initial stages of in vitro LDL oxidation, "minimally" modified LDL (mmLDL) without altered apo B, is capable of recruiting inflammatory cells via the induction of monocyte chemotactic protein-1 synthesis in smooth muscle and endothelial cells (57-59). Mice lacking the receptor for monocyte chemotactic protein-1 are resistant to atherosclerosis (60, 61). With more extensive oxidation, the apo B component is fragmented and lysine residues are covalently modified, which results in the direct recruitment of monocytes (62) and T lymphocytes (63) due in part to the formation of lysophosphatidylcholine (64). This recruitment of inflammatory monocytes and T cells further contributes to the continued oxidation of LDL, thus setting the stage for the progression of the atherosclerotic lesion. In addition to producing these chemotactic factors, the

oxidation of LDL also promotes the proliferation of smooth muscle cells (65), the production of autoantibodies (66, 67), and the formation of immune complexes that further promote the uptake of LDL into macrophages (68, 69). Together these processes form the events necessary for the initiation and progression of atherosclerosis.

While each of these three hypothesis (the respose-to-injury, the response-to-retention, and the oxidative modification model) recognizes that LDL is the primary factor involved in the initiation and progression of atherosclerosis, and that a humoral inflammatory response is critical for the progression of the disease, the oxidative modification hypothesis is unique in its emphasis of the importance of oxidative events in initiating atherogenesis. In order to better understand the oxidative modification model and the impact that it may have on our understanding of atherosclerosis, it is important that we examine the factors that influence LDL oxidation. By doing so, we may be able to elucidate the mechanisms by which LDL oxidation is regulated, which could potentially enable us both to better predict the progression of atherosclerotic disease, as well as direct pharmacologic or gene based therapies to alter this process.

What Controls LDL Oxidation?

There are many oxidants that have been shown in the laboratory to have the potential to form reactive oxygen species (ROS) or reactive nitrogen species (RNS). These two products have been shown to play important roles in LDL oxidation, and their *in vivo* production results from a variety of cellular and extracellular sources as well as from the utilization of both enzymatic and nonenzymatic pathways. The sources of these oxidants are reviewed in detail by Stocker and Keaney (1), and include NAD(P)H oxidases, xanthine oxidase, nitric oxide synthase (NOS) enzymes, myeloperoxidase, lipoxygenases, free transition metals including iron and copper, lipid peroxyl radicals, and the superoxide anion radical ($O_2^{-\cdot}$) produced as a by-product of electron flow through the mitochondrial electron transport chain. The work presented in this

thesis builds upon the importance of ROS generated by monocyte mitochondria, and potential regulatory factors in this process.

There is growing evidence that ROS production via mitochondrial respiration may play an important role in inflammation, and that increases in mitochondrial membrane potential can enhance mitochondrial superoxide generation (70-73). Work in this area has also suggested that uncoupling proteins (UCPs), as a result of their ability to regulate proton leakage through the inner mitochondrial membrane, may be key regulators of mitochondrial ROS production (70, 74-76).

UNCOUPLING PROTEINS

Mitochondrial uncoupling proteins are members of the mitochondrial anion carrier superfamily that includes UCP1, UCP2 and UCP3. UCPs are integral mitochondrial membrane proteins with a tripartite structure consisting of 6 α-helical regions spanning the lipid bilayer with both the amino and carboxyl termini on the cytosolic side, and long hydrophilic loops on the mitochondrial matrix side. UCPs function to dissipate the electrochemical proton (H^+) gradient generated by mitochondrial respiration which drives ATP production via the F_0F_1-ATPase. UCP-1, originally named thermogenin, was discovered in 1976 from functional studies of brown adipose tissue mitochondria and was one of the earliest membrane proteins to be sequenced. UCP1 was originally thought to be a unique protein found only in mammalian brown adipose tissue, but over the past 30 years further research has found five other uncoupling proteins in humans, and another one that is found in plants.

UCP1 is found exclusively in brown adipose tissue and is involved in non-shivering thermogenesis. It was cloned in the orthodox manner in that the physiological phenomena of non-shivering thermogenesis was first identified, then a cellular explanation was discovered (uncoupled mitochondria in brown adipose tissue), then a responsible protein was identified (UCP1), and lastly the mRNA corresponding to the protein was sequenced (77). Conversely,

UCP2 and UCP3 were identified using "reverse cloning", they were identified from databases of expressed sequence tags or from cDNA libraries as a result of their similarity to UCP1. As a result, the functions of UCP2 and UCP3 were unknown at the time of their identification in 1997. Since then, there has been considerable research aimed at identifying the role of these proteins, although a consensus has not yet been reached. It is clear from the levels of expression of UCP2 and UCP3 that the heat produced by their uncoupling would not be physiologically significant. Based in part on this finding, it is believed that the functions of UCP2 and UCP3 are not the same as UCP1.

Since their identification 7 years ago, there have been several functional roles suggested for UCP2 and UCP3. Multiple studies have demonstrated that UCP3 mRNA is found almost exclusively in brown adipose tissue and skeletal muscle (78-81). Given this distribution, it has been suggested that UCP3 is associated with energy metabolism and regulation of the metabolic rate. In a study of Pima Indians, it was found that a polymorphism within the proximal promoter region of UCP3 is associated with an increased resting metabolic rate which predisposes affected individuals to having a lower total body weight (82).

UCP2 on the other hand, has been shown to be present throughout the body including in the spleen, liver, skeletal muscle, heart, brain, and peripheral mononuclear cells (77). As a result, there have been numerous suggested roles for UCP2 (77). These include the regulation of mitochondrial ATP synthesis, regulation of fatty acid metabolism, and regulation of ROS production and ROS detoxification.

This work focuses on the proposed roles that UCP2 plays in the regulation and production of ROS, and the relevance that this has in the development of atherosclerosis from the perspective of the oxidative modification model for atherogenesis.

UCP2 and Reactive Oxygen Species

The superoxide radical ($O_2^{\cdot-}$), which serves as the primary precursor to mitochondrial H_2O_2 generation, is produced largely as a natural by-product of mitochondrial respiration. The ability of UCP2 to regulate the production of H_2O_2 was first shown by Negre-Salvayre et al. who found that GDP was able to raise membrane potential and H_2O_2 production of mitochondria from nonparenchymal cells expressing UCP2, but was unable to do so in hepatocytes devoid of UCP2 (74). Further work investigating the relationship between UCP2 and ROS production demonstrated that macrophages from UCP2-/- knockout mice have greater superoxide and ROS production than wild type animals (83). Additionally, higher levels of ROS have been shown to be present in mice deficient for UCP3 (79).

While first hypothesized in 1997 (74), the correlation between the regulation of mitochondrial ROS production by UCP2 and the oxidation of LDL leading to the progression of atherosclerotic disease was not demonstrated experimentally until 6 years later when Blanc et al. demonstrated that UCP2 plays a protective role in the development of atherosclerosis (84). Their experiments demonstrated that when irradiated low-density lipoprotein receptor-deficient (LDLR-/-) mice received a bone marrow transplant from either wild-type or UCP2-/- donor mice and were subsequently fed an atherogenic diet, there was a significant difference in both plaque size and plaque composition depending on the genotype of the donor mouse. Specifically, in LDLR-/- mice that received bone marrow transplants from UCP2-/- mice there was a 42% increase in atherosclerotic plaque size in the aortic sinus, and a 93% increase in the percentage of plaque surface in the thoracic aorta as compared to mice receiving marrow from UCP2+/+ mice (84). These experiments emphasize the importance of UCP expression in marrow derived hematologic cells in regulating atherogenesis.

UCP and Atherosclerosis

Further investigations into the mechanisms behind this proposed inverse relationship between UCP expression and atherosclerotic disease progression have suggested that not only does increased UCP expression result in decreased ROS production (74, 79), but also that UCPs can inhibit monocyte transendothelial migration, thereby inhibiting the accumulation of monocytes/macrophages in an atheromatous lesion (85). Work by Ryu et al. demonstrated that the decreased ROS production that occurs as a result of monocyte UCP2 overexpression resulted in less monocyte Ca^{++} influx, which subsequently lowered monocyte expression of the ß-integrin, CD11b, by 75 to 80%. CD11b is a regulator of monocyte recruitment and adhesion to endothelial cells, and by decreasing its surface expression by overexpressing UCP2, Ryu et al. found that there was an inhibition of β_2 integrin-dependent adhesion of the monocytes to endothelial cells (85). Ryu et al. went on to demonstrate that overexpression of UCP2 in monocytes also inhibited the migration of monocytes across cultured endothelial cells in response to monocyte chemoattractant protein-1.

Taken together these findings suggest that, through its ability to decrease ROS production, UCP2 may both inhibit the oxidation of LDL, critical for the progression of atherosclerosis as detailed in the oxidative model, and also prevent the recruitment and migration of monocytes across the endothelium.

PURPOSE

To determine if there is an association between mononuclear cell UCP2 expression and the severity of coronary artery disease in patients undergoing cardiac catheterization.

HYPOTHESES

Decreased UCP2 expression in mononuclear cells results in increased reactive oxygen species production, and thus increases LDL oxidation, resulting in more severe coronary artery disease. Conversely, increased UCP2 expression in mononuclear cells provides less opportunity for LDL modification, and thus confers greater resistance to atherosclerotic plaque formation.

SPECIFIC AIMS

1. To obtain patient whole blood samples, isolate peripheral mononuclear cells, and purify mRNA.
2. To develop and validate an assay to measure monocyte UCP2 mRNA expression.
3. To analyze patient coronary angiograms.
4. To perform statistical analysis to determine the relationship between monocyte UCP2 expression and the severity of coronary artery disease as measured by the stenosis score.

METHODS

The author of this thesis participated in all aspects of the design of the study, recruitment of patients, collection of patient data, collection and preparation of blood samples, performance of real time-RT-PCR analysis, review of coronary angiograms, and data analysis. The following groups and individuals contributed to the conduct of the studies: attending invasive cardiologists of Yale University School of Medicine and Yale-New Haven Hospital (performed the coronary angiography), and J. Dawn Abbott, MD, Kerry Strong Russell, MD, PhD and Raymond Russell, MD, PhD (reviewed the coronary angiograms in a blinded fashion, the author was present for all of the angiogram reviews). All aspects of the investigation were supervised by Raymond Russell, MD, PhD.

1. Patient Selection

All patients older than 17 years of age who were referred to the Yale-New Haven Hospital Cardiac Catheterization Laboratory for diagnostic cardiac catheterization were eligible for the study. Exclusion criteria included the inability to give informed consent, self-reported blood transfusion within the previous 14 days of the procedure, history of heart transplantation, self-reported acute inflammatory/febrile illness, self-reported autoimmune disease, self-reported acute bacteria or viral infection within the previous 14 days, self-reported chronic infections (e.g., chronic hepatitis, HIV), self-reported autoimmune disorders, symptomatic anemia, treatment with immunomodulatory agents, or use of thyroid hormone replacement agents, which are known to affect UCP expression. Patients were approached to request participation in the study prior to the time of their cardiac catheterization. This occurred on the outpatient unit, on the medical wards, and in the catheterization laboratories of Yale-New Haven Hospital. Patients were given ample time to read and have the protocol explained to them as well as to ask questions. The protocol was approved by the Human Investigation Committee of the Yale University School of Medicine.

2. Collection of Patient Information

Patient information including age, sex, race, height, weight, current medications, allergies, smoking history, family history of heart disease, history of diabetes, history of previous coronary revascularization, and history of congestive heart failure where gathered by direct patient questioning and review of the patients' medical record. Laboratory data including serum glucose and creatinine concentrations, lipid profile, complete blood count and left ventricular ejection fraction, when available, were recorded. (See Appendix 1 for sample patient information sheet).

3. Collection of Patient Blood Samples

During the catheterization procedure, blood was collected prior to angiography via the peripheral arterial sheath that was placed as part of the cardiac catheterization. The blood was then distributed into 4 tubes: 10ml each into 2 heparin coated tubes, 9ml into a tube containing SST® Gel and Clot Activator for serum isolation, and 9ml into a tube containing 3.2% buffered sodium citrate for plasma isolation. The samples were kept at 4°C for between 1 and 4 hours before being processed in the laboratory.

4. Acquisition and Analysis of Coronary Angiograms

Coronary angiography was performed using standard techniques. Coronary artery angiograms were either digitally acquired and stored on the Yale-New Haven Hospital Cardiac Catheterization Laboratory server, or recorded onto film, depending on the catheterization lab in which the procedure was performed.

Angiograms were review by three board certified cardiologists (J. Dawn Abbott, MD, Kerry Strong Russell, MD, PhD and Raymond Russell, MD, PhD) experienced at reading

coronary angiograms who were blinded to the patient's clinical and biochemical information. The burden of coronary atherosclerotic disease was assessed by the stenosis score described by Sullivan et al. (86). In this method, the coronary arteries are divided into 15 segments and each segment is scored visually using a 0-4 scale for the presence of stenosis (0: no stenosis, 1: <25% stenosis, 2: 25-50% stenosis, 3: 51-75% stenosis, 4: >75% stenosis). The stenosis score is then calculated as the sum of the scores for each of the 15 segments. (See Appendix 2 for sample stenosis scoring sheet).

5. Isolation of Peripheral Blood Mononuclear Cells

Mononuclear cells were isolated using Sigma Diagnostics Accuspin tubes following the protocol that accompanied the tubes. First, Histopaque-1077 was brought to room temperature and 15 ml of Histopaque-1077 was pipetted into the upper chamber of the Accuspin tube. The tube was then centrifuged for 30 seconds at room temperature, after which time the Histopaque was displaced into the chamber below the "frit". Blood from both of the heparin coated tubes (20ml total) was then poured into the upper chamber of the Accuspin Tube. Ten milliliters of sterile phosphate buffered saline (PBS) was combined with the blood. The tube was then centrifuged at 800 x g and 18-26°C for 15 minutes. After centrifugation, the yellow plasma layer was aspirated with a 25 ml pipet to within 0.5 cm of the opaque "buffy coat" containing mononuclear cells, and the plasma was discarded. The "buffy coat" was then transferred into a 50 ml conical tube using a P1000 pipette whose tip had been cut off to prevent unnecessary trauma to the cells. The mononuclear cells were then washed by adding 10 ml of isotonic PBS and resuspending the cells in the PBS with a 25 ml pipette. The sample was then centrifuged at 250 x g at 18-26°C for 10 minutes, and then washed again and centrifuged as above. The cells were then resuspended in 1 ml of PBS and divided into 500 µl aliquots that were places in 1.5-ml microfuge tubes. These tubes were then spun at 5000 rpm in a microfuge at room temperature for 5 minutes. The supernatant was then aspirated off and the microfuge tubes containing the cell

pellets were inverted on drying paper for 2 minutes. To one tube, 300 µl of freezing media was added, the cells were resuspended, and the sample stored at -80 °C to cryopreserve cells for functional studies and DNA analysis that are to be performed at a later date. To the other microfuge tube, 600 µl of RLT buffer (Qiagen) was added to the cells in preparation for RNA isolation.

6. RNA Isolation from Peripheral Blood Mononuclear Cells

Messenger RNA was isolated from peripheral blood mononuclear cells using Qiaqen RNeasy kits. After resuspension in 600 µl of RLT buffer, the cells were homogenized by carefully passing the lysate 8 times through a 20-gauge needle fitted to an RNase-free syringe. Ethanol (70%, 600 µl) was then added to the homogenized lysate and mixed by pipetting. Six hundred microliters of this sample, including any precipitate that had formed, was then added to an RNeasy mini column placed inside a 2-ml collection tube. The tube was closed and centrifuged for 15 sec at 8000 x g.

The flow-through from the RNeasy mini column was discarded, and the remaining sample was loaded onto the same RNeasy column and centrifuged as before. After centrifugation, the flow-through was discarded and 350 µl of buffer RW1 was added to the RNeasy mini column containing the sample, and centrifuged for 15 seconds at 8000 x g. The flow-through was discarded and on-column DNase digestion was performed using Qiagen RNase-free DNase Set to remove any genomic DNA that could interfere with subsequent quantification of the mRNA. Per the product protocol, 80 µl of DNase I incubation mix (containing 10 µl DNase I stock solution and 70 µl buffer RDD) was pipetted directly onto the RNeasy silica-gel membrane, and incubated at room temperature for 15 minutes. Three hundred-fifty microliters of Buffer RW1 were then added to the RNeasy mini column, which was centrifuged at room temperature for 15 seconds at 8000 x g. The RNeasy column was transferred to a new 2 ml collection tube, and 500 µl of Buffer RPE was added to the column. The tube was

closed and centrifuged for 15 seconds at 8000 x g, and the flow-through discarded. Another 500 µl of Buffer RPE was then added to the tube and centrifuged for 2 minutes at 8000 x g to dry the RNeasy silica-gel membrane. The RNease mini column was then carefully removed from the collection tube and placed into new 1.5 ml collection tube which was centrifuged for an additional to 2 minutes at 8000 x g to ensure that there was no residual flow-thorough. This column was placed into a new 1.5 ml collection tube, and 50 µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The tube was closed and centrifuged for 1 minute at 8000 x g. The RNeasy column was then discarded and the 1.5 ml microfuge tube containing the flow-through with isolated RNA was stored at -80 °C.

7. Reverse Transcription of RNA

RNA concentrations were determined by mixing 5 µl of isolated RNA with 95 µl of water and measuring the absorbance of the resultant mixture using a Spectromic BioMate 3 spectrophotometer at a wavelength of 260 nm. An 11 µl aliquot of RNA was then prepared at a concentration of 1 µg/11 µl in a 1.5 µl microfuge tube. For each reverse transcription reaction, 1 µl of Promega olgio$(dT)_{15}$ primer was added to the 11 µl aliquot and incubated at 70°C for 10 minutes. The mixture was then placed on ice, microfuged at 8000 x g for 20 seconds, and returned to ice. A reverse transcriptase mastermix containing 4 µl of 5X first strand synthesis buffer (Gibco) per reaction, 2 µl of 0.1M DTT (Gibco) per reaction, and 1 µl of 10mM dNTPs (Gibco) per reaction was then prepared, and 7 µl of the mastermix was added to the microfuge tube containing the RNA and oligo$(dT)_{15}$ primer. This reaction mixture was incubated at 42 °C for 2 minutes, after which 1 µl of Superscript II (Gibco) was added. This was then incubated at 42 °C for 50 minutes, followed by incubation at 70 °C for 15 minutes. RNase H (0.6 µl, Gibco) was then added and the mixture was incubated at 37 °C for 20 minutes. The total volume of the reaction was then brought to 100 µl by the addition of 79 µl of water and heated to 100 °C for 10

minutes. Immediately afterward, the reaction mixture, which contained reverse transcribed cDNA, was placed at -80 °C for storage.

8. Real-Time Analysis of cDNA

UCP2 mRNA expression levels were determined using quantitative real time RT-PCR amplification with Taqman probes. The primer and probe sequences used for the analysis are as follows: UCP2 forward primer CTACAAGACCATTGCCCGAGA; UCP2 reverse primer TGTTGCTCGTAATGCCATTGT; UCP2 probe HEX-TGGGAGAGGTCCTTTCCAGAGGCC. A standard curve was produced from pooled normal control mRNAs. Reactions were performed using 1x Taqman master mix (ABI), 200nmoles of each primer, 100nmoles of the probe, and 2.5µl of the mRNA reverse-transcription product (cDNA) in a total volume of 25µl. The mRNA expression levels of glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were measured using TaqMan Pre-developed Human GAPDH Assay Reagents (Applied Biosystems part #4333764F) containing both primers and probe for human GAPDH according to the manufacturer's instructions. For each sequence amplified, triplicates of no template control and standard curve samples were amplified at the same time for quality control.

Reactions were run on an MJ Research DNA Engine Opticon 2 continuous fluorescence detector real time instrument according to the following thermocycle protocol: 1. 50°C for two minutes; 2. 95 °C for ten minutes; 3. 95 °C for fifteen seconds; 4. 58 °C for one minute; 5. read plate and repeat the process starting at step 3 fifty more times. The threshold cycle (Ct) values of the triplicate samples were checked to ensure that they were within 1 Ct of each other.

Data obtained from the real time quantitative PCR was analyzed using the $2^{-\Delta\Delta C}{}_T$ method as developed by Livak and Schmittgen (87) and recommended by the manufacturer (Qiagen). The $2^{-\Delta\Delta C}{}_T$ method allows for the relative quantification of the expression level of the target gene (UCP2) in a certain population relative to a reference group. Using this method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene

(GAPDH) and relative to a control group. For the control group, $\Delta\Delta C_T$ equals zero and 2^0 equal one, so that the fold change in gene expression relative to the control equals one, by definition. For the non-control groups, evaluation of $2^{-\Delta\Delta C}{}_T$ indicates the fold change in gene expression relative to the control group.

In our analysis, we choose several different reference groups, and compared changes in UCP2 expression using this technique. For example, in looking at the correlation between UCP2 expression and coronary artery disease, patients with no to mild coronary disease (the lowest tertile of patients with stenosis score 0-10) were chosen as the reference group. Using the $2^{-\Delta\Delta C}{}_T$ method thus allowed for relative quantification of UCP2 expression in patients with moderate coronary disease (middle tertile of patients, stenosis score 11-25) and severe disease (highest tertile, stenosis score>25) as a fold change when compared to the UCP2 levels in the control group. In analyzing the correlation between changes in UCP2 expression and body mass index (BMI), patients with a normal BMI (18.5-24.9) where chosen as the control group, thus allowing for the relative quantification of UCP2 expression in overweight (BMI 25-29.9) and obese (BMI $\geq$30) patients as compared to the control group. In analyzing the correlation between changes in UCP2 expression and the presence of diabetes, diabetics patients were identified either by an established diagnosis of diabetes in the medical record, the use of antidiabetic agents or a fasting plasma glucose concentration >125 mg/dl.

10. Statistical Analysis

All patient data were entered into a database (Filemaker Pro) for further analysis. Results are reported as the mean ± standard error of the mean, or number (%) of patients. Differences between continuous variables were assessed by Student's t-test, for categorical variables a chi-square analysis was used. A p value <0.05 was considered statistically significant.

RESULTS

Patient Population Results

A total of 103 qualified patients were entered into the study, mRNA was successfully isolated and reverse transcribed into cDNA for real-time analysis, and coronary angiograms were reviewed. Relevant demographic and clinical data were collected (Table 1). This population consisted of 72 (70%) men and 31 (30%) women, and the mean age of the patients was approximately 67 years (age range: 32-86 years). The population was 90% Caucasian with one Asian, three Hispanic, and seven African American patients. Twenty-two percent of patients were current smokers, 42% had a family history of heart disease, and 67% had a history of hypercholesterolemia (defined by a fasting total cholesterol >200 mg/dl or currently on lipid lowering therapy). The average body mass index (BMI) was 29.6 kg/m^2, and by breakdown, 20% of the patients were classified as having a normal weight (BMI <25), 48% as being overweight (BMI 25-30), and 32% as being obese (BMI >30), based on World Health Organization criteria.

Table 1: Demographic and Clinical Characteristics

	Overall (n=103)	Low stenosis score (n=31)	High stenosis score (n=35)
Age (y)	66.7±12.3	58.6±11.4	70.3±11.4
Male (%)	70%	42%	91%*
Body mass index (kg/m^2)	29.6±7.6	32.4±10.7	28.2±4.6
Diabetic (%)	33%	26%	43%
Hypertensive (%)	65%	61%	71%
Tobacco use (%)	22%	13%	26%
Hypercholesterolemic (%)	67%	58%	71%
Family history (%)	42%	42%	49%

Values reported as the mean±standard deviation or %. *$p<0.005$.

Generation of Stenosis Score

The stenosis scores for patients were calculated based on the sum of the atherosclerotic disease severity scores for 15 distinct segments of the coronary arteries (86) (See Appendix 2 for sample stenosis scoring sheet). The scores for our patient population ranged from zero (figure 3A) to 60 (figure 3B).

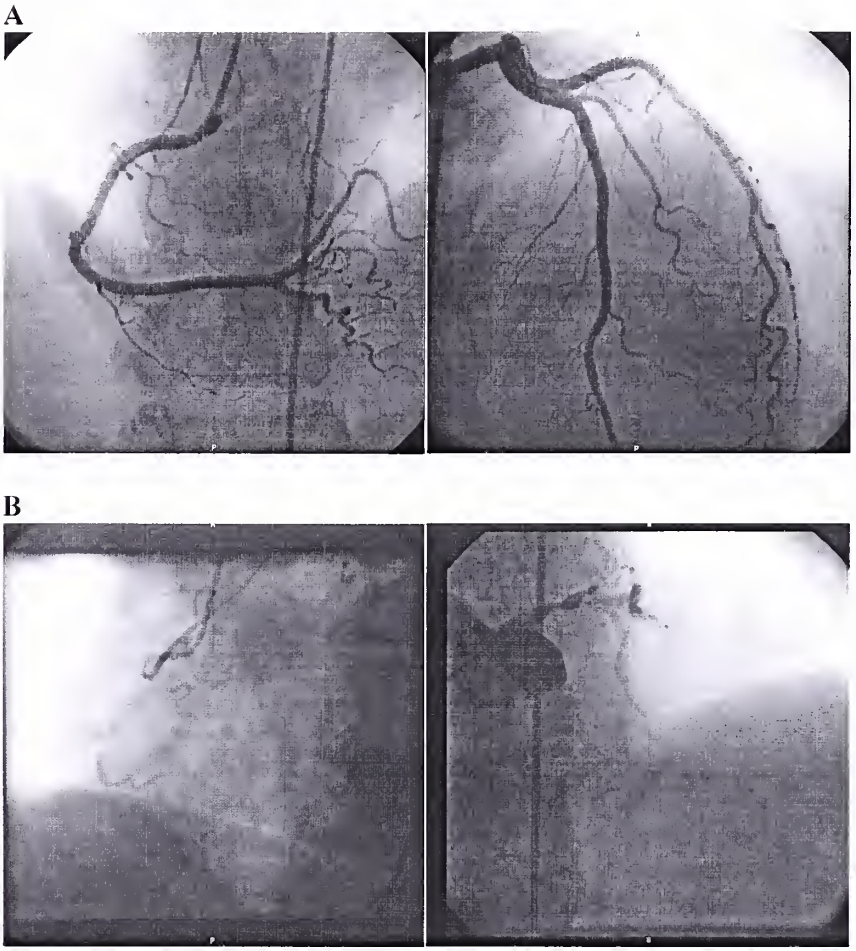

Figure 3. Sample Coronary Angiograms.

A. Representative angiograms of the coronary arteries from a patient with no disease (control group). Right coronary system (top left panel) and left coronary system (top right panel).

B. Representative angiogram of the coronary arteries from a patient with multiple, severe stenoses of the coronary arteries (severe disease group). Right coronary system (bottom left panel) and left coronary system (bottom right panel).

Distribution of Stenosis Scores

The distribution of stenosis scores in our patient population (figure 4) showed a skewing towards patients with lower stenosis scores. Scores ranged from zero to sixty, with a mean of 20, a median of 18, and a mode of 0. This skewing is likely due the population referred for diagnostic cardiac catheterization based on the results of previous stress testing.

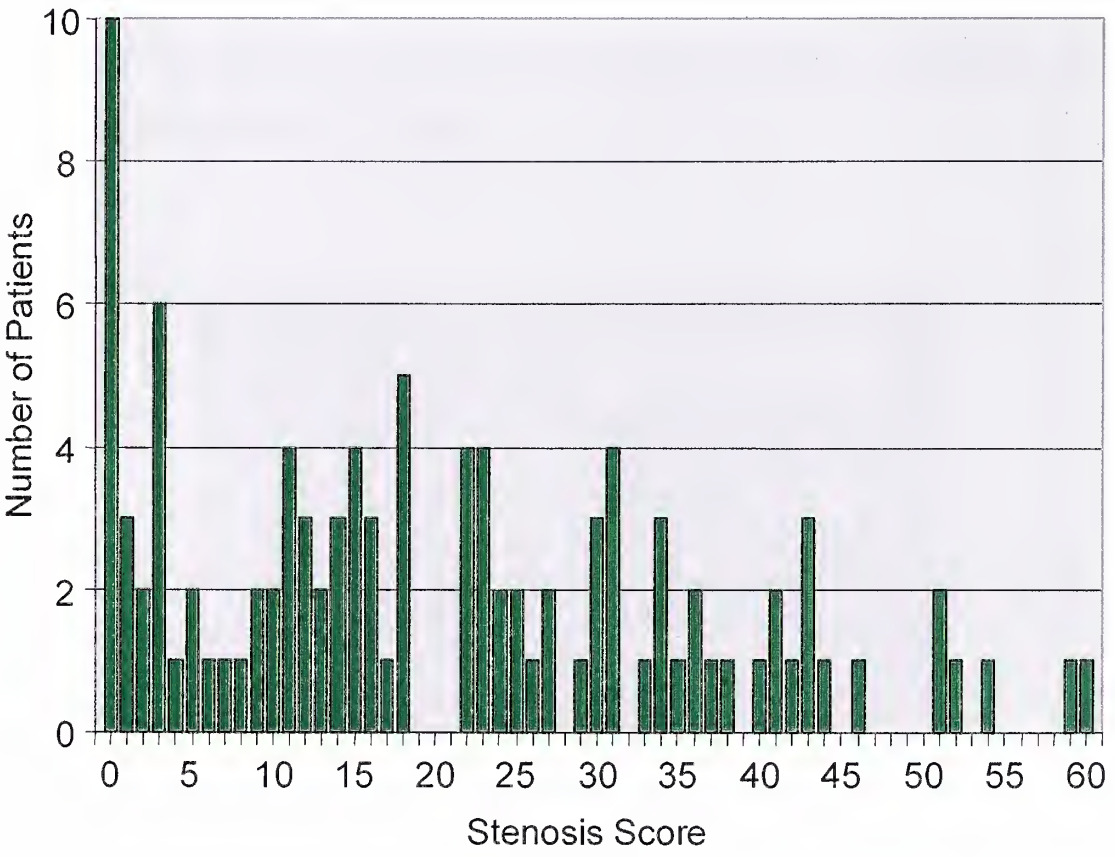

Figure 4. Distribution of Stenosis Scores in the Study Population.
Amongst the 103 patients enrolled in our study there was a skewing towards lower stenosis scores. The mean stenosis score was 20.

Validation of the $2^{-\Delta\Delta C_T}$ Method Using GAPDH as the Endogenous Reference Gene

To assess the validity of the $2^{-\Delta\Delta C_T}$ method as a means to determine the relative quantification of UCP2 gene expression, varying amounts of cDNA were subjected to RT-PCR and a standard curve for the resultant GAPDH C_T was generated and compared to a standard curve for UCP2 C_T. In order to use GAPDH as our reference gene, it was necessary to confirm that the amplification efficiencies of UCP2 (target gene) and GAPDH (reference gene) were similar, thus controlling for RNA input amount. The PCR efficiencies for GAPDH and UCP2 were similar with an efficiency slope for GAPDH of -3.63 and for UCP2 -3.70 (figure 5), thus validating the use of GAPDH as our endogenous reference gene for determining relative UCP2 expression levels using the $2^{-\Delta\Delta C_T}$ method.

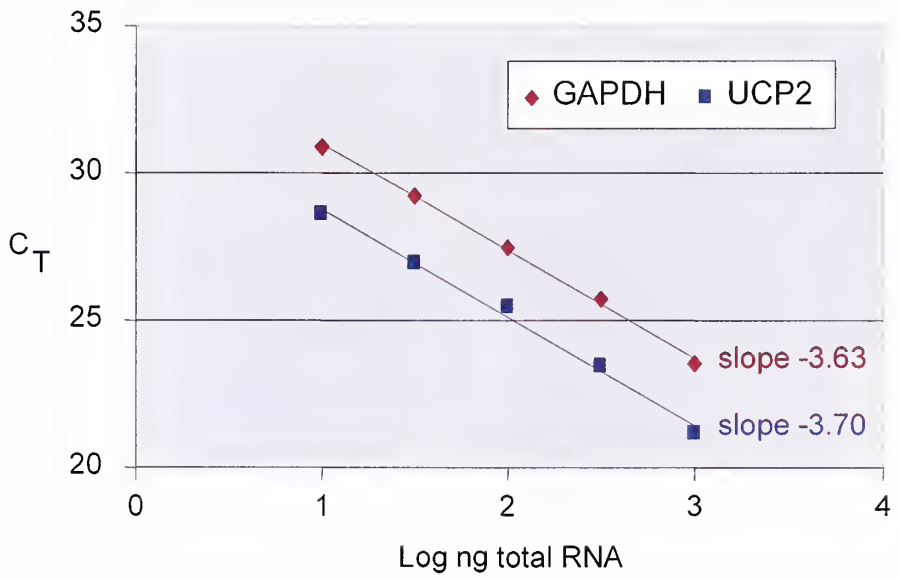

Figure 5. Quantitative Detection of UCP2 and GAPDH mRNA by Real Time PCR. Data represents output Ct values versus total input RNA amount. GAPDH and UCP2 show similar real-time PCR reaction efficiencies as determined by the slopes of the lines.

Relationship Between Monocyte UCP2 Expression and Atherosclerotic Disease

Based on their angiographic stensosis score, patients were categorized as having either no-to-mild coronary atherosclerotic disease (stenosis score 0-10, n=31), moderate disease (stenosis score 11-24, n=37), or severe coronary disease (stenosis score ≥ 25, n=35). Real-time PCR analysis of patient samples was then performed using the $2^{-\Delta\Delta C}{}_T$ method, and changes in the expression of the target gene UCP2 were normalized to GAPDH. There was no difference seen between mean UCP2 expression levels in monocytes from the control group (no to mild coronary disease) and the moderate disease group. However, there was a 35% decrease in mean UCP2 expression in the patients in the severe group when compared to the no to mild coronary disease group ($p<0.02$, figure 6). These findings indicate that there is a relationship between monocyte expression of UCP2 and the atherosclerotic burden of the coronary arteries.

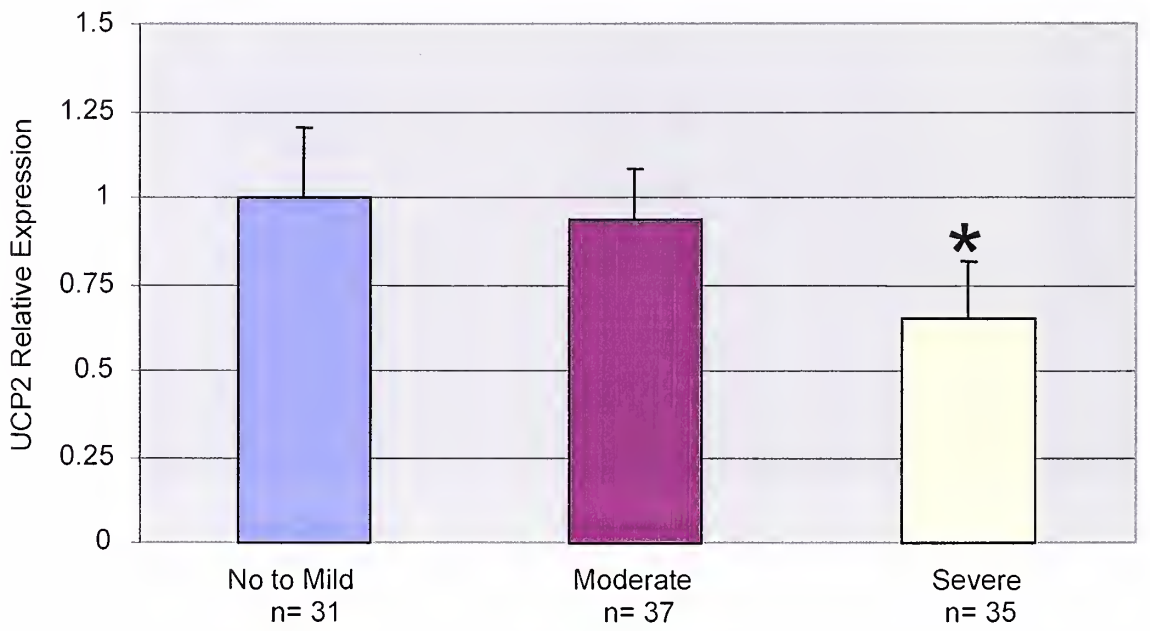

Figure 6. Correlation of UCP2 Expression and Coronary Artery Disease.
Average relative UCP2 expression values of patients divided into tertiles based on coronary artery atherosclerotic disease burdern. First tertile, no to mild stenosis (stenosis score 0-10); second tertitle moderate stenosis (stenosis score 11-24); and third tertile, severe stenosis (stenosis score >25). Patients with more severe atherosclerotic disease were found to have a 35% ($p< 0.02$) reduction in their UCP2 mRNA expression levels.

Relationship Between Monocyte UCP2 Expression and Body Mass Index

Previous studies have demonstrated that polymorphisms within the promoter region of UCP2 and UCP3 lead to increased mRNA expression in skeletal muscle and are associated with changes in body mass (82, 88, 89). Therefore, we examined whether there is also an association between monocyte UCP2 expression and obesity. Patients were categorized into three groups based on their BMI: normal weight (BMI 18.5-24.9, n=20), overweight (BMI 25-30, n=49), or obese (BMI > 30, n=30). In our analysis of the relationship between BMI and UCP2 expression, although there was a trend toward an increase in monocyte UCP2 expression with increasing BMI, the difference was not statistically significant (figure 7).

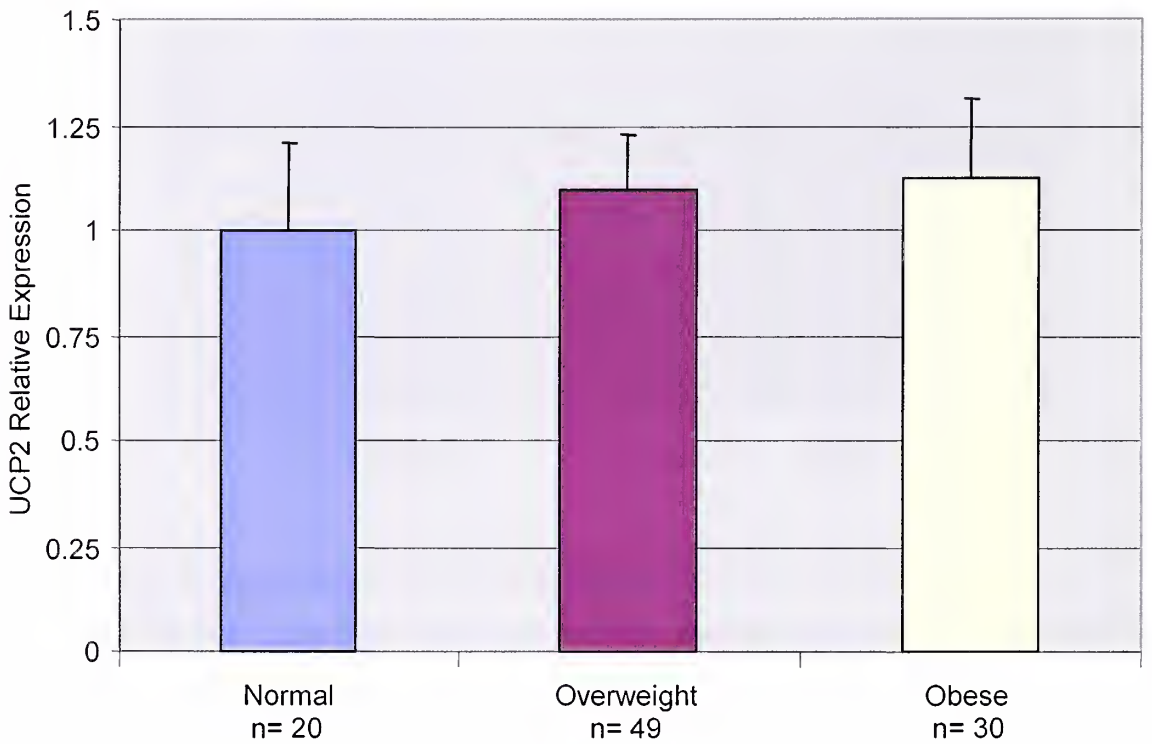

Figure 7. Correlation of UCP2 Expression and Body Mass Index.
Average relative monocyte UCP2 expression values in patients categorized as being of normal weight (BMI 18.5-24.9), overweight (BMI 25-30), or obese (BMI >30). There was no significant difference observed between the three subsets.

Relationship Between Monocyte UCP2 Expression and Diabetes

Previous studies have demonstrated that polymorphisms within the promoter region of UCP2 are associated with a decreased risk of diabetes (89, 90). Therefore, we compared UCP2 gene expression between nondiabetic patients (n=69) and those with diabetes mellitus (n=34). In our analysis of the relationship between the incidence of diabetes and monocyte UCP2 expression, we found no significant difference between the two groups (figure 8).

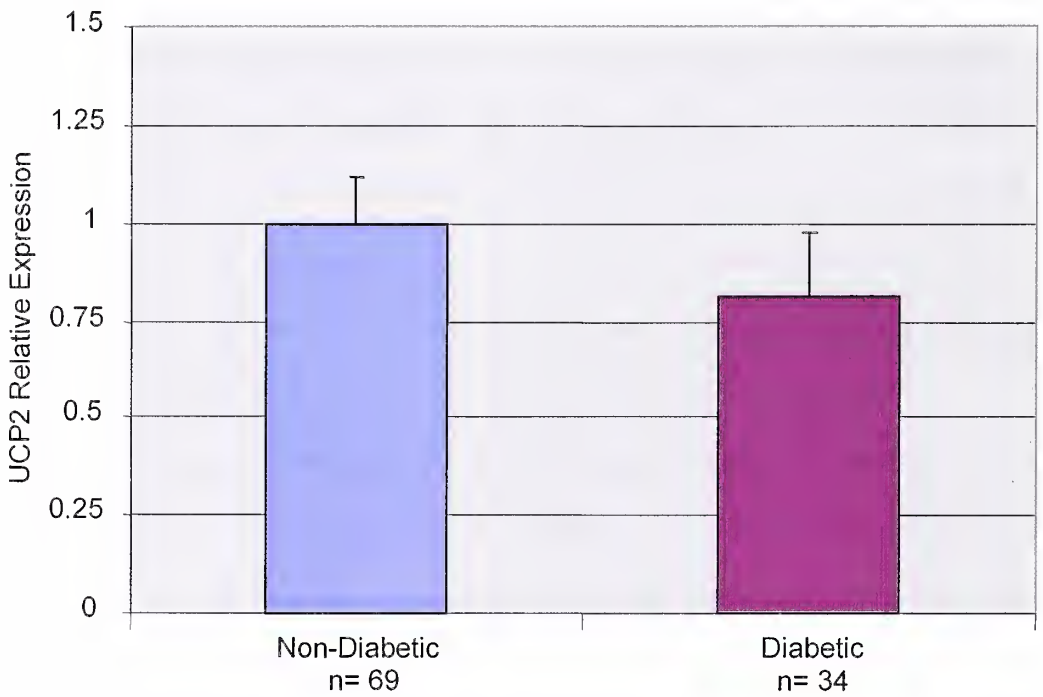

Figure 8. Correlation of UCP2 Expression and Diabetes.
Average relative UCP2 expression values of non-diabetic and diabetic patients. There was not a statistically significant difference observed between the two groups.

DISCUSSION

This work represents the first known study undertaken to examine the relationship between uncoupling protein expression and atherosclerotic disease burden in patients. While previous work examining the proposed functions of UCPs has indicated that these proteins may influence atheromatous plaque formation and progression as a result of their regulation of reactive oxygen species production, our findings in this study are the first to demonstrate in a clinical setting that decreased monocyte UCP2 expression is associated with the presence of severe coronary artery disease in humans. This finding represents an important advancement in our understanding in the pathophysiology of atherosclerotic disease. By identifying UCP2 as a potential regulator of atherogenesis, there exists not only the possibility that UCP2 gene expression analysis could be used as a marker for risk stratification, but also that pharmacologic or genetic manipulation of UCP2 expression might help to inhibit atherogenesis.

The mechanism by which decreased UCP2 expression promotes atherosclerosis is likely related to the influence that uncoupling proteins have in the regulation of reactive oxygen species production (74, 79, 91). Reactive oxygen species have been hypothesized to play an important role in the oxidation of native-LDL into its atherogenic form, and also have been shown to inhibit the adhesion of monocytes to the endothelium and transendothelial migration (70, 85, 92, 93). The importance of monocyte migration and adhesion to the endothelium lining the coronary vessels is recognized as a critical step in atherosclerotic plaque formation, and disruption of this process could inhibit plaque formation under otherwise atherogenic conditions. Furthermore, the oxidative modification theory of atherogenesis suggests that the oxidation of native-LDL results in chemical alterations which make the LDL molecule susceptible to uptake by macrophages, thus promoting formation of the "fatty streak" and allowing for the propagation of the atherosclerotic plaque. By limiting this process through creating an environment with decreased oxidant stress, UCP2 could further prevent the development of severe coronary artery disease.

A critical aspect of this study is that we examined UCP2 mRNA expression not from coronary endothelium, but rather from isolated peripheral blood mononuclear cells. The rationale for this stems from the work by Blanc et al. who demonstrated that the extent and severity of atherosclerotic plaque formation in irradiated LDLR-/- mice differs depending on whether they received a bone marrow transplant from wild type or UCP2-/- mouse (84). Since irradiated mice who received bone marrow from UCP2-/- mice developed more severe atherosclerotic disease as compared to mice who received wild-type marrow, it can be concluded that differences in circulating cells, generated by the transplanted marrow, are in part responsible for this difference in disease progression. One limitation of our experiment with respect to the role of circulating monocytes, is that we isolated all of the peripheral mononuclear cells (including lymphocytes) from the blood. We chose this approach in part because monocyte-specific isolation is significantly more difficult, involving flow cytometric isolation based on cell surface markers, and would not haven been feasible for such a large patient sample size, and also because it is known that lymphocytes play a critical role in the inflammatory mechanisms of atherosclerosis, and thus their level of UCP2 might also be an important factor. However, if a more practical method for monocyte isolation were to become available, it would be worthwhile to broaden this study by investigating the expression level of UCP2 in isolated monocytes and isolated lymphocytes.

While we did not investigate UCP2 mRNA expression in the coronary endothelium due to the invasive and difficult nature of isolating such samples, future studies determining the role of endothelial UCPs in protection against oxidant stress are of significant interest. The potential for UCP2 to alter monocyte adhesion and transendothelial migration secondary to reduced ROS and H_2O_2-induced ROS production, suggests that UCP2 expression in endothelial cells might also play a role in protection against atherogenesis. Future work to investigate this possibility would likely be limited initially to animal models, but would provide further insight into the physiologic function of uncoupling proteins in the vascular system.

Our data examining the relationship between UCP2 expression, obesity, and diabetes failed to reveal a significant relationship. Other studies, however, have identified polymorphisms in the promoter of the human UCP2 gene that are associated with changes in insulin secretion, the risk of diabetes, and obesity (88-90, 94). These studies focused on the -866G/A polymorphism in the promoter region of the UCP2 gene and demonstrate that the A/A polymorphism results in increased UCP2 mRNA expression in skeletal muscle and is associated with a decreased risk of obesity but increased susceptibility to diabetes. One explanation for why we were unable to observe such a correlation between UCP2 expression, obesity, and diabetes, is that our study group does not represent the generation population, but rather a small subgroup of patients with symptomatic coronary artery disease. Additionally, despite the high frequency (47%) of the A/A polymorphism, previous studies have demonstrated this polymorphism accounts for only 15% of the risk for obesity (88). Furthermore, those studies measured skeletal muscle UCP2 mRNA expression, which is likely regulated by different transcription factors than those in monocytes. Future studies involving our patient cohort are planned to identify the patients who have the A/A polymorphism, and to compare the frequency of obesity and diabetes, in addition to the severity of atherosclerosis, with the frequency of the genotypes (G/G, G/A and A/A) in those subjects.

Another limitation of this work is that we are only measuring moncyte UCP2 mRNA expression at a single point in time. Since the majority of patients who were enrolled in this study were older and were undergoing coronary angiography for clinical indications, we were not able to investigate the possibility that the monocyte UCP2 expression might have been different during the early stages of atherogenesis when levels of LDL oxidation may be crucial to the initiation and early progression of the disease. Similarly, one cannot exclude the possibility that the decreased UCP2 expression we observe here is not the cause, but rather the result of severe coronary disease. While this is unlikely, one method of investigating this possibility would be to conduct a prospective study looking at the development of coronary disease in a large cohort of patients to determine whether UCP2 expression levels measured in asymptomatic patients is

predictive of the severity of the coronary artery disease that develops later. Another approach that could be taken to determine the cause and effect relationship between UCP2 expression and coronary disease would be to determine UCP2 expression in the offspring of affected patients who have low levels of UCP2 expression to study the heritability of UCP2 expression levels. Decreased UCP2 expression in pre-symptomatic offspring of individuals with low levels of UCP2 expression would support the notion that decreased UCP2 levels are not a consequence of atherosclerotic disease.

As previously suggested, the hypothesis that decreased monocyte UCP2 expression plays a role in the development of atherosclerotic disease raises the possibility that we might be able to alter this prevalent process through pharmacologic or genetic manipulation of UCP2 expression. If the oxidation of LDL by reactive oxygen species (generated as a by-product of electron flow though the mitochondrial electron transport chain) is a critical step in the initiation and progression of atherosclerotic disease, as has been suggested, then increasing UCP2 expression would likely decrease the production of these ROS and thus protect again disease development. While gene therapies for diseases such as cystic fibrosis have been slow to develop, the reason for this stems from the fact that the cells in which you are trying to alter expression are exceedingly difficult to manipulate for a variety of reasons. In cystic fibrosis, the respiratory epithelium is the affected cell type, but since part of its inherent function is to keep foreign particles, including gene transfer vectors, out of the lungs, gene therapy has been very difficult. In contrast, genetic manipulation of peripherally circulating mononuclear cells is much easier to achieve, thus allowing for the possibility of UCP2 alterations.

Additionally, by needing to only be distributed in the peripheral blood, there would exist the opportunity to develop pharmacologic agents that could be administered orally and could alter the regulation of UCP2 expression. Currently, there are four agents that are known to increase UCP2 expression in skeletal muscle and liver: the AMPK activator 5-aminoimidazole-carboxyaminde-1-ribofuranoside (AICAR), PPAR-α and PPAR-γ agonists, and thyroid hormone

(95-99). It remains to be determined whether these agents have any effect on the expression of UCP2 in monocytes.

As with any genetic or pharmacologic modification, the effects of increasing UCP2 expression would need to be more carefully elucidated before such alterations were made, as the result of increased expression could also have negative effects. In particular, since ROS also play an important role in the body's response to infection, severe downregulation of their production by over-expression of UCP2 might alter immune function. In fact, targeted gene disruption studies have shown that as compared to wild-type mice, UCP2-/- knockout mice are completely resistant to *Toxoplasma gondii*, while such infections are lethal in the wild-type mice (83).

In summary, we have shown that decreased UCP2 mRNA expression is associated with an increased burden of coronary artery disease in humans. This finding opens the door to a new area of research for both atherosclerotic disease risk stratification and prevention. With further understanding of the function of uncoupling proteins, there exists the potential for better prevention and recognition of atherosclerotic disease burden, an advance that could substantially improve the ability of physicians to treat this disease which affects so many of our patients.

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APPENDIX 1: SAMPLE PATIENT INFORMATION SHEET

Code | Last name | First name | MRUN | Date

Age | Gender ☐M ☐F | Ht (m) | Wt (kg) | LVEF

Race: ☐African American ☐Hispanic ☐Asian ☐Caucasian ☐Native American ☐Pacific Islander

Indication

☐Acute MI ☐CSA ☐CHF
☐USA ☐Chest pain/+stress test ☐Valvular disease

Risk Factors

DM	☐Yes	☐No
HTN	☐Yes	☐No
TOB	☐Yes	☐No
Fam Hx	☐Yes	☐No
Hyperchol	☐Yes	☐No
Prev CAD	☐Yes	☐No
CHF	☐Yes	☐No

Meds

ASA	☐Yes	☐No
Statin	☐Yes	☐No
Niacin	☐Yes	☐No
Fibrate	☐Yes	☐No
ß-blocker	☐Yes	☐No
CCB	☐Yes	☐No
ACE-I	☐Yes	☐No
ARB	☐Yes	☐No
Plavix	☐Yes	☐No
Insulin	☐Yes	☐No
TZD	☐Yes	☐No
Metformin	☐Yes	☐No
Sulfonylureas	☐Yes	☐No
Heparin	☐Yes	☐No
GP IIbIIIa	☐Yes	☐No

Treatment

☐No therapy
☐Medical therapy
☐PTCA/Stent
☐CABG

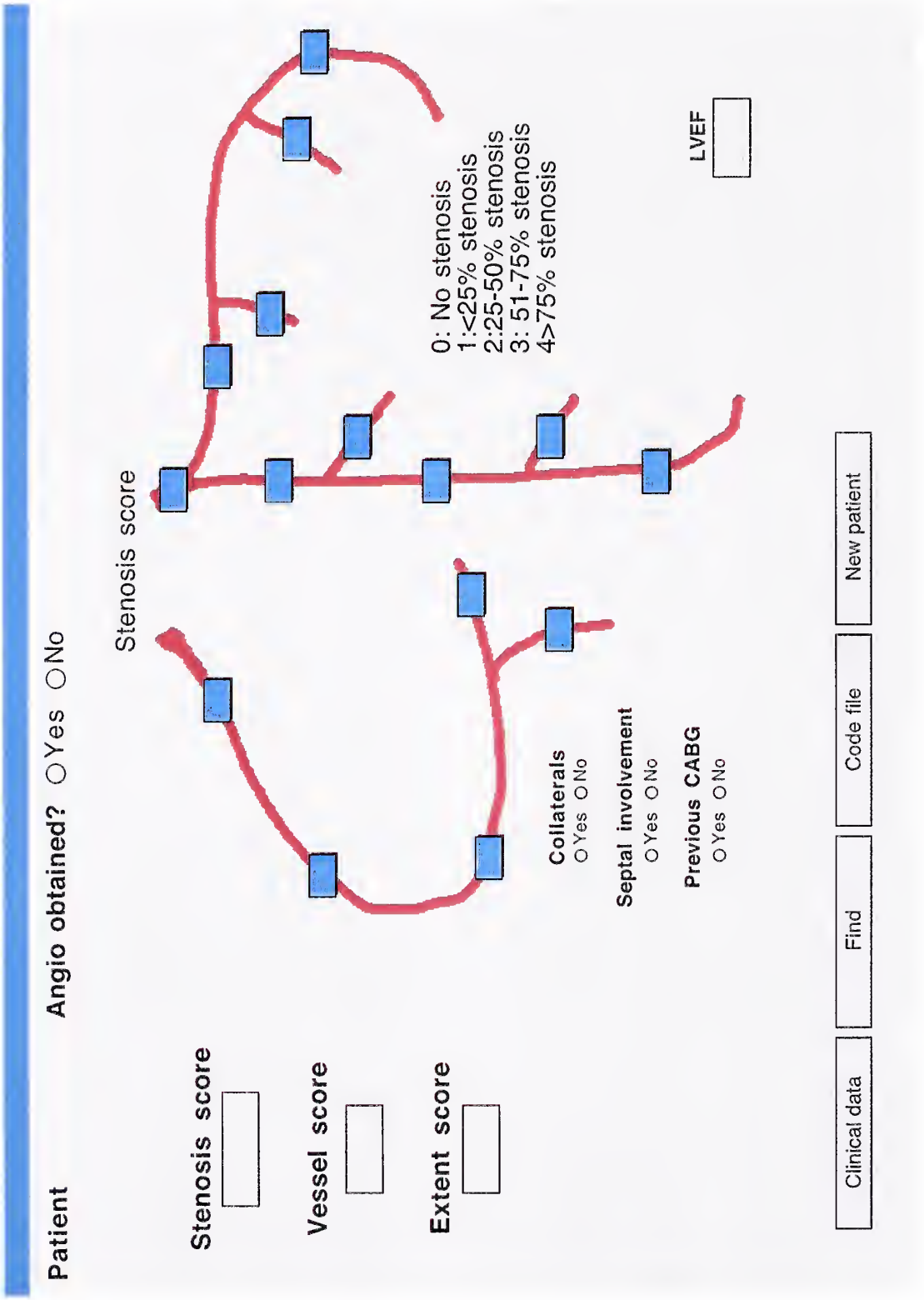
Restenosis? ☐Yes ☐No

Glucose | Creat | Tot chol | HDL | LDL | TGLY

WBC | HGB | PLT

Other drugs:

APPENDIX 2: SAMPLE STENOSIS SCORING SHEET



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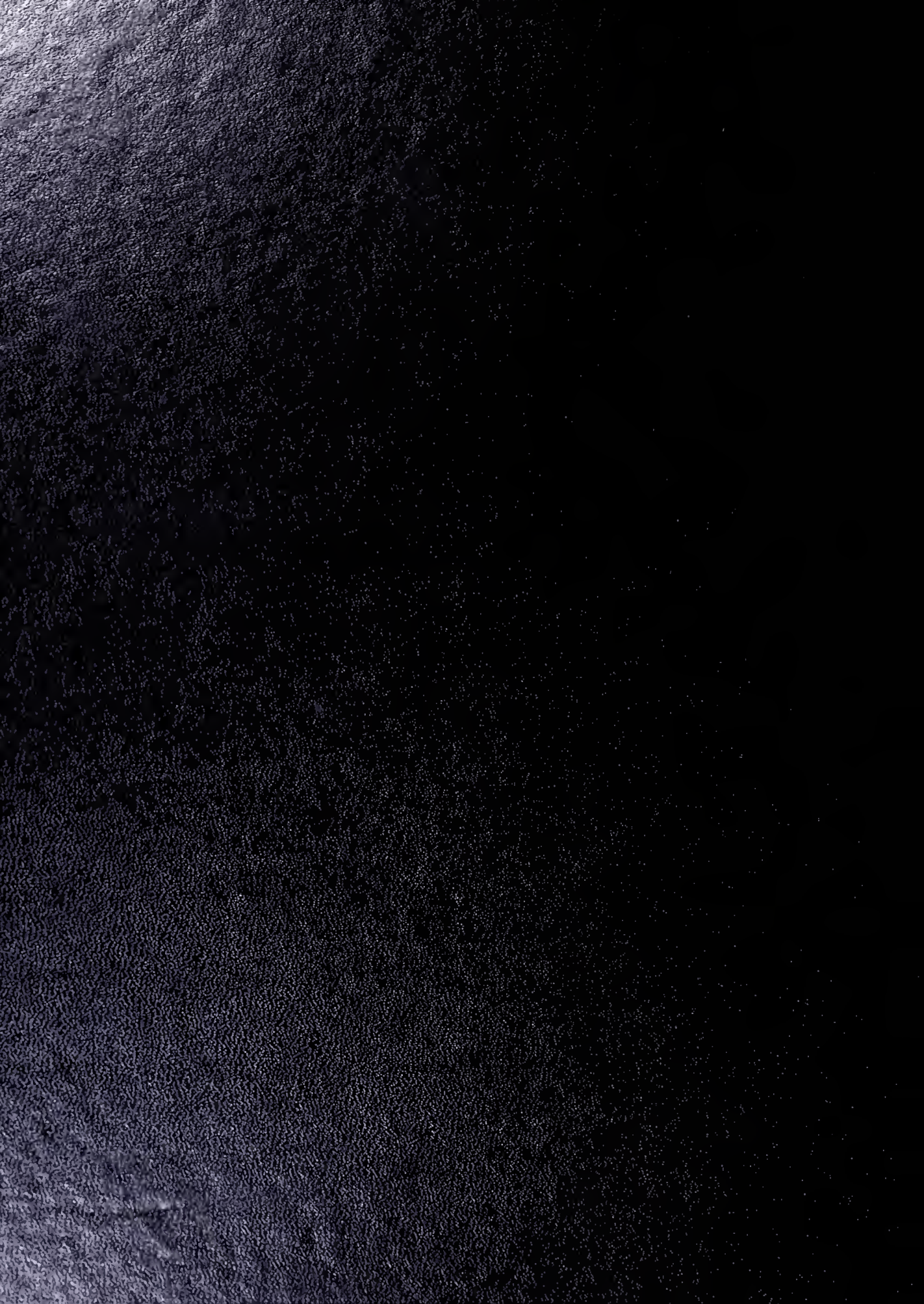
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